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*J Immunol* 2009; 182:2221-2230; doi: 10.4049/jimmunol.0801878

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Enhanced In Vivo Growth of Lymphoma Tumors in the Absence of the NK-Activating Receptor NKp46/NCR1

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The in vitro elimination of virus-infected and tumor cells by NK cells is regulated by a balance between signals conveyed via specific inhibitory and activating receptors. Whether NK cells and specifically the NK-activating receptor NKp46 (NCR1 in mice) are directly involved in tumor eradication in vivo is still largely unknown. Since the NKp46/NCR1 tumor ligands have not been identified yet, we use a screening technique to identify functional ligands for NKp46/NCR1 which is based on a cell reporter assay and discover a NCR1 ligand in the PD1.6 lymphoma line. To study whether NKp46/NCR1 is important for the eradication of PD1.6 lymphoma in vivo, we used the Ncr11/1/1 mice generated by our group. Strikingly, all Ncr1 knockout mice developed growing PD1.6 tumors, whereas initial tumor growth was observed in the wild-type mice and tumors were completely rejected as time progressed. The growth of other lymphoma cell lines such as B10 and EL4 was equivalent between the Ncr1 knockout and wild-type mice. Finally, we show that PD1.6 lymphoma cells are less killed both in vitro and in vivo in the absence of NKp46/NCR1. Our results therefore reveal a crucial role for NKp46/NCR1 in the in vivo eradication of some lymphoma cells.


It was hypothesized that the immune system surveys the body for nascent malignancies, eliminating some tumors and slowing the growth of others (1). This hypothesis is supported by the findings that humans with congenital or acquired immunodeficiency have a significantly higher incidence of malignancies (2, 3). Consistent with such a role, various components of the immune system, such as CTLs, NK cells, and Abs can exert potent activity against different types of tumors in vitro (4–6).

NK cells are bone marrow-derived lymphocytes of the innate immune system comprising ~5–15% of PBLs. NK cells play an important role in the early elimination of virus-infected cells, bacteria, intracellular parasites, and tumor cells (7–14). In addition, NK cells have been shown to also perform several noncytotoxic functions, including: Ag presentation (15), activation of dendritic cells (16), an important regulatory function in the maternal-fetal interface (17), and secretion of immunomodulatory cytokines such as IFN-γ, TNF-α, and chemokines causing T cells to shift to a TH1 phenotype (18).

The antitumor cell activity of NK cells was the hallmark of their original discovery (19). However, only a limited number of in vivo studies investigated whether NK cells in general and NK receptors in particular are involved in tumor eradication. Studies involving depletion of NK cells in mice often showed reduced resistance to transplanted tumor cell lines (20–22). Subsequent studies demonstrated that mice carrying the homozygous beige mutation, which are defective in granule exocytosis and have a profound defect in NK cell cytolytic function, develop virus-induced and carcinogen-induced tumors at a higher frequency (23–25). However, these studies did not prove a role for NK cells in tumor immunity, since the defects caused by the beige mutation are not limited to NK cells. Other studies demonstrated an increased rate of carcinogen-induced sarcomas (and of tumors induced by oncogenic viruses) in mice lacking perforin, the pore-forming granule protein involved in cytotoxicity by NK cells and CTLs (26). In contrast to perforin-deficient mice, mice genetically deficient for CD8+ T cells did not show significant defects in the control of carcinogen-induced tumor growth, suggesting a possible role of NK cells in tumor surveillance (26). Importantly, perforin-deficient mice also developed spontaneous disseminated lymphomas at a much higher frequency than immunocompetent control mice (27). Whether the perforin dependence of tumor resistance in normal mice reflects the activity of NK cells or T cells, or both, was not determined in this system.

NK cell’s activation is governed by numerous receptors (some of which are activating while others are inhibitory) that recognize various classes of cell surface ligands (14, 28). The balance between these countervailing signals tightly regulates NK cell activation (14, 28). Many of the inhibitory receptors expressed by NK cells are specific for MHC class I molecules as predicted by the “missing self-recognition” hypothesis (29) (although other, non-MHC-restricted receptors were also identified (30–32)). In contrast, activating NK cell receptors recognize self, stress-inducible, pathogen-derived, and tumor ligands, whose expression is induced or up-regulated in target cells (14, 28). In that respect, a significant breakthrough in understanding NK cell activation was made following the identification of three novel, NK-specific activating receptors, including NKp30, NKp44, and NKp46 and its mouse ortholog NCR1 (33–35), which are collectively known as natural cytotoxicity receptors.
(NCRs). Among the NCRs, NKp46 and its mouse ortholog NCR1 are constitutively expressed by NK cells and, in fact, are the best marker which distinguishes NK cells from other immune cells in all tested mammals, including humans (35), mice (36, 37), monkeys (38), rats (39), and bovines (40).

Several in vitro studies have demonstrated that NKp46 in humans and NCR1 in mice are major killer receptors involved in the recognition and killing of tumor cells (5, 41). In keeping, a low concentration of NKp46 has been observed in several types of malignancies, including acute myeloid leukemia (42). We have previously demonstrated that viral hemagglutinins (HA) of various influenza viruses are recognized by NKp46 and NCR1 (14, 37, 43, 44); however, a cellular tumor ligand for NKp46 or NCR1 still remains to be identified.

To study the in vivo function of NKp46, we have previously generated a knockout mouse in which a GFP cassette was “knocked in” into the Ncr1 locus, thereby rendering Ncr1 non-functional (Ncr1<sup>gfp/gfp</sup>) (37). As we have previously reported, influenza infection was lethal in Ncr1<sup>gfp/gfp</sup> mice in both 129/Sv and C57BL/6 backgrounds, demonstrating that the recognition of HA by NKp46/NCR1 is essential for influenza virus eradication in vivo (37). That being said however, the direct role of NKp46/NCR1 in the elimination of tumor cells in vivo has not been clarified. The results obtained in these mice regarding tumor growth were much less conclusive, as we have observed reduced clearance of the RMAS tumor cells in vivo in Ncr1<sup>gfp/gfp</sup> mice in the 129/Sv background but not in the C57BL/6 background (37).

In this study, we identified an unknown ligand for NKp46/NCR1 expressed on the PD1.6 lymphoma line and show, both in vitro and in vivo, that eradication of PD1.6 lymphoma is impaired in the absence of NCR1. Finally, we demonstrate that the mechanism by which NK cells, and NKp46/NCR1 specifically, are involved in the eradication of the PD1.6 lymphoma cells is direct cytotoxicity rather than secretion of IFN-γ.

Materials and Methods

Mice and in vivo tumor experiments

All experiments were performed using mice 6–8 wk old of the C57BL/6 background. The generation of NKp46/NCR1 knockout mice in the C57BL/6 background was previously described (37). All of the experiments reported herein were performed in a specific, pathogen-free unit of the Hebrew University Medical School (Ein-Kerem, Jerusalem) in accordance with the guidelines of the ethics committee. For in vivo tumor growth monitoring, mice were injected s.c. with 1 × 10<sup>6</sup> tumor cells in a volume of 200 μl of sterile PBS. Tumor size was measured every 1–2 days after injection using a micrometer. Experiments were repeated three to four times. For the detection of NK cells and T cells in tumors, mice were injected s.c. with 1 × 10<sup>6</sup> tumor cells and sacrificed 11 days later when tumors were removed and analyzed. Experiments were repeated twice.

Cells

The cell lines used in this study were: for the in vivo study, we used the C57BL/6-derived murine radiation leukemia virus-induced thymic lymphomas PD1.6 and B10, the murine thymoma EL4. In addition, we used the murine mastocytoma P815, the human epithelial carcinoma HeLa, the murine thymoma BW, the RMAS lymphoma, and the murine lymphoma YAC-1. The P815, BW, PD1.6, EL4, B10, and YAC-1 cell lines were all grown in complete RPMI medium that was supplemented with 10% FCS and 0.05 mM βME. The HeLa cell line was grown in complete DMEM medium that was supplemented with 10% FCS.

Fusion proteins, Abs, and flow cytometry

The NCR1-Ig, NKp46-Ig, NKp46 D1-Ig, and NKG2D-Ig fusion proteins were generated in COS-7 cells and purified by affinity chromatography using a protein G column, as previously described (44). The staining of cell lines was visualized using a secondary PE-conjugated goat anti-human Ab (Jackson ImmunoResearch Laboratories). Abs used in this work included: a hybridoma-producing mAb 12CA5 (anti-HA tag), a polyclonal Ab against mouse NKp46 AF2225 (R&D Systems), staining of both Abs was visualized using fluorescein-conjugated goat anti-mouse Ab and fluorescein-conjugated donkey anti-goat Ab (Jackson ImmunoResearch Laboratories), respectively. PE-conjugated anti-CD3 Ab, and negative control mouse IgG1/PE (DakoCytomation). Additionally, a hybridoma-producing mAb PK136 (anti-NK1.1) was used for the depletion of NK cells in vivo.

BW assay

For measurements of IL-2 production resulting from the interaction between NCR1 and ligands expressed by target tumor cell lines, 50,000 BW or BW/NCR1-Δ cells were coincubated with 50,000 irradiated (3000 rad) cells of various mouse cell lines for 48 h at 37°C and 5% CO₂. Supernatants were collected and the level of IL-2 was quantified by using commercially available anti-mouse IL-2 mAbs (BD PharMingen) and standard ELISA.

NK cell depletion

Fifty micrograms of anti-NK1.1 Ab in a volume of 300 μl of sterile PBS was injected i.v. into the tail vein of C57BL/6 Ncr1<sup>gfp/gfp</sup> mice. Sterile PBS was injected as a control. Depletion was verified 2 days after injection: mice were bled; RBCs were lysed (using ACK buffer), and GFP-positive cells were analyzed by flow cytometry.

In vitro cytotoxicity assays

Heterozygous and Ncr1-deficient C57BL/6 mice were injected i.p. with 200 μg of poly(I):poly(C) (Sigma-Aldrich). Spleens were removed 18 h later and NK cells were isolated from extracted splenocytes using a mouse NK isolation kit (Miltenyi Biotec) and an AutoMACS instrument according to the manufacturer’s instruction. NK cells (5 × 10<sup>4</sup>) were coincubated with the indicated target cells in triplicate at a ratio of 1:1 in the presence of 0.1 μg of a allophycocyanin-conjugated anti-CD107a Ab (1D4B; Southern Biotechnology Associates). Following a 2-h incubation at 37°C and 5% CO₂, cells were washed and analyzed by flow cytometry.

In vivo lung clearance assay

For the in vivo cytotoxicity assay, we used a published fluorescence labeling method with a modification to avoid possible influence of intrinsic fluorescence. NKp46<sup>+/gfp</sup> or BW/NCR1<sup>+/gfp</sup> mice (37, 45). Cells were labeled with Vybrant green (Molecular Probes). Subsequently, HeLa cells were labeled with Vybrant DiD; PD1.6 cells were labeled with Vybrant DiI. Cells (2 × 10<sup>6</sup>) of each cell type were mixed in 400 μl of sterile PBS and injected into the tail vein of C57BL/6 Ncr1<sup>Δ/+</sup> or Ncr1<sup>GFP/gfp</sup> mice. Lungs, spleen, liver, lymph nodes, and kidneys were explanted either immediately after injection or 3 h later. Single-cell suspensions were obtained by using cell strainers and fluorescence was analyzed by flow cytometry. The ratio of the tested PD1.6 target cells to the internal control HeLa cells was calculated.

Cytokine secretion assay

Heterozygous Ncr1<sup>Δ/+</sup> and NCR1-deficient Ncr1<sup>GFP/gfp</sup> C57BL/6 mice were injected i.p with 200 μg of poly(I):poly(C) (Sigma-Aldrich). Spleens were removed 18 h later and NK cells were isolated from extracted splenocytes using a mouse NK isolation kit (Miltenyi Biotec) and an AutoMACS instrument according to the manufacturer’s instruction. Fifty thousand NK cells were coincubated with the indicated target cells at a ratio of 1:1 for 48 h at 37°C and 5% CO₂. Supernatants were collected and IFN-γ levels were determined with a commercially available anti-mouse IFN-γ mAb (BD PharMingen) and standard ELISA.

Results

Mouse lymphoma cell lines express ligand(s) for NKp46/NCR1

The importance of NK cells in general and that of NKp46 in particular in the in vitro killing of many tumors is well established (28, 46, 47). We have previously demonstrated that NCR1, the

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3 Abbreviations used in this paper: NCR, natural cytotoxicity receptor; HA, hemagglutinin.
FIGURE 1. Expression of NKp46/NCR1 ligands on mouse tumor cell lines. A–C, Binding of NCR1-Ig (A), NKp46-Ig (B), or NKp46 D1-Ig (C) fusion proteins to various mouse tumor cell lines. Gray shading, background staining with secondary Ab only; dark lines, specific staining with the indicated fusion protein. The various tumor cell lines are indicated. Data from one of four independent experiments are shown. D, Flow cytometry analysis of BW cells expressing HA-tagged NCR1ζ chimeric protein using anti-HA tag and anti-NCR1 mAbs. Gray shading, background staining with secondary Ab only; dark line, specific staining with the indicated Ab. Data from one of three independent experiments are shown. E, IL-2 secretion by BW or BW/NCR1-ζ cells following a 48-h incubation with several mouse tumor cell lines. Values are mean ± SD for triplicate samples. Data from one of three independent experiments are shown. F, IL-2 secretion from BW or BW/NCR1-ζ cells following a 48-h incubation with target cell lines in the presence or absence of NCR1-Ig fusion protein at a concentration of 5 μg/sample. Values are mean ± SD for triplicate samples. Data from one of two independent experiments are shown.
mouse ortholog of NKP46, plays a critical function in the in vivo eradication of influenza virus (37). However, its function in the in vivo killing of tumor cells has remained unclear (37). Since the cellular ligands for NKP46 are yet unknown, we studied whether NKP46 or its mouse ortholog (NCR1) recognizes ligand(s) expressed by mouse tumor cell lines using Ig fusion proteins. For this purpose, the extracellular portion of NKP46 receptor or the extracellular portion of NCR1 receptor was fused to the Fc portion of human IgG1, as previously described (37, 44). As a negative control, we used the truncated extracellular portion of NKP46 missing the receptor binding domain NKP46 D1-Ig (47). The binding of the various fusion proteins to various mouse tumor cells lines was tested in flow cytometry analysis (Fig. 1, A–C). Several mouse lymphoma cell lines, including PD1.6, B10, and EL4, were recognized by NCR1-Ig fusion protein (Fig. 1A), suggesting that they express ligand(s) for NCR1. Other cell lines tested, such as BCL1, AML, 66.3, and LB, were not recognized (data not shown). Furthermore, all cell lines that were shown to express ligands for NCR1 were also stained by NKP46-Ig fusion protein (Fig. 1B). The fact that there is cross-reactivity between human and mouse NKP46 receptor and that mouse tumor cell lines are recognized also by NKP46-Ig is not surprising because it was previously shown that NKP46 and NCR1 ligands share great homology (14, 37). The binding of the various fusion proteins to various mouse tumor cells lines was tested in flow cytometry (Fig. 1, A–C). Several mouse lymphoma cell lines, including PD1.6, B10, and EL4, were recognized by NCR1-Ig fusion protein (Fig. 1A), suggesting that they express ligand(s) for NCR1. Other cell lines tested, such as BCL1, AML, 66.3, and LB, were not recognized (data not shown). Furthermore, all cell lines that were shown to express ligands for NCR1 were also stained by NKP46-Ig fusion protein (Fig. 1B). The fact that there is cross-reactivity between human and mouse NKP46 receptor and that mouse tumor cell lines are recognized also by NKP46-Ig is not surprising because it was previously shown that NKP46 and NCR1 ligands share great homology (14, 37). The NKP46-Ig and NCR1-Ig staining was specific, as no staining was observed with the control fusion protein NKP46 D1-Ig (Fig. 1C). Additionally, the tested cell lines were stained using NKG2D-Ig fusion protein to test whether they express NKG2D ligands and, if so, whether there is a difference in the expression level of these ligands between the cell lines. The result obtained showed low expression of NKG2D ligands on all tested cell lines, with no marked difference in their expression level (data not shown).

**NCR1 is efficiently activated by PD1.6**

We next wanted to evaluate which of the cell lines that were shown to express ligands for NCR1/NKP46 will efficiently activate these receptors. To that end, we have used the BW reporter system. BW cells are a mouse thymoma cell line lacking the expression of the TCR α- and β-chains, which can be induced to secrete IL-2. The BW cells also express an unknown ligand for NCR1, as demonstrated by flow cytometry analysis of BW cells stained with the NCR1-Ig, NKP46-Ig, and the negative control NKP46 D1-Ig fusion proteins (Fig. 1, A–C). An NCR1-ζ chimeric protein, composed of the extracellular portion of the NCR1 receptor fused to the mouse ζ-chain, was constructed and stably expressed in BW cells. The NCR1-ζ construct also contains a HA-tag to detect expression on the cell surface. As can be seen in Fig. 1D, expression of the chimeric protein on BW cells was verified by both anti-NCR1 and anti-HA tag Abs. In this system, the triggering of the receptor fused to the ζ-chain by its ligand will lead to the secretion of IL-2. Indeed, since BW cells express an unknown ligand for NCR1 (Fig. 1, A and B), secretion of IL-2 from BW/NCR1-ζ cells was observed even without any target cells, whereas little or no IL-2 self-secretion was observed from BW cells alone (Fig. 1E). Next, we wondered which of the tumor cell lines, recognized by NCR1-Ig and NKP46-Ig (Fig. 1, A and B), will be able to induce IL-2 secretion from BW/NCR1-ζ that could overcome the effect mediated by the endogenous NCR1 ligand expressed by BW cells. BW or BW/NCR1-ζ cells were incubated with the various mouse tumor cell lines that were shown to express ligand(s) for NCR1 (Fig. 1A) and IL-2 level was quantified in cell supernatants by ELISA 48 h later. Ligation of the NCR1-ζ with anti-HA tag Ab induced a strong IL-2 production which exceeded the basal IL-2 secretion from BW/NCR1-ζ (Fig. 1E), indicating that this assay could be used to detect functional ligands for NCR1. Such a ligand was shown to exist on PD1.6 cells as a significant 2-fold increase

**FIGURE 2.** Enhanced PD1.6 tumor growth in Ncr1<sup>-/−</sup> mice. Tumor size was monitored every 1–2 days after s.c. injection of 1 × 10<sup>6</sup> PD1.6 cells in six heterozygous Ncr1<sup>+/+</sup> mice (■) and six NCR1-deficient Ncr1<sup>-/−</sup> mice (□). Summary of the average tumor size is shown. Data from one of four independent experiments are shown.

**FIGURE 3.** Enhanced PD1.6 tumor growth in NK-depleted mice. A, Flow cytometry analysis of peripheral blood cells derived from mice injected with anti-NK1.1 Ab (right plot) or with sterile PBS only (left plot) to detect GFP-positive NK cells. Percentages of GFP-positive cells are indicated in the plots. B, Tumor size was monitored every 2 days following s.c. injection of 1 × 10<sup>6</sup> PD1.6 cells in five NK-depleted heterozygous Ncr1<sup>+/+</sup> mice (■) and five untreated heterozygous Ncr1<sup>+/+</sup> mice (□). Summary of average tumor size is shown.
in the secretion level of IL-2 was observed when these cells were coincubated with BW/NCR1- \( \text{H9256} \) cells (Fig. 1E), whereas incubation of PD1.6 cells with untransfected BW cells resulted in little or no IL-2 secretion (Fig. 1E). The increased production of IL-2 was due to the interaction of the unknown ligand, expressed by PD1.6 cells, with the NCR1 receptor, as it was blocked by NCR1-Ig fusion protein (Fig. 1F). Taken together, these results suggest that PD1.6 cells were the only cells tested that express a potent functional ligand for NCR1.

Enhanced in vivo PD1.6 tumor growth in the absence of NCR1

To test the in vivo function of NK cells in general and of NKp46/NCR1 in particular in tumor eradication, we used the NKp46/NCR1-knockout Ncr1\(^{gfp/gfp}\) mice that we have generated (37). Heterozygous Ncr1\(^{gfp/+}\) or NCR1-deficient Ncr1\(^{gfp/gfp}\) mice in the C57BL/6 background were injected s.c. with \( 1 \times 10^6 \) PD1.6 cells and tumor growth was monitored every 1–2 days. Remarkably, in the absence of NKp46/NCR1, tumor eradication was impaired. In both the Ncr1\(^{gfp/+}\) mouse group and the Ncr1\(^{gfp/gfp}\) mouse group, tumor growth was visible beneath the skin by day 5 and measurable around day 7 following injection (Fig. 2). In all Ncr1\(^{gfp/+}\) mice tested, tumors started to appear from day 7 onward, reached a maximum average size of 65 mm\(^2\) on day 11, then began to diminish and were not visible throughout the end point of the experiment (day 24; Fig. 2). In marked contrast, in all Ncr1\(^{gfp/gfp}\) mice, tumor growth was gradual, reaching \( \sim 400 \) mm\(^2\) by day 24, which was when mice were sacrificed due to ethical reasons (Fig. 2). These results demonstrate that PD1.6 tumor growth in vivo is NKp46/NCR1 dependent.

**NK cells are responsible for initial control of tumor growth**

A role for NK cells has been shown not only in the direct killing of tumor cells during the initial phase of the innate antitumor response, but also in the activation of T cells and the regulation of the adaptive immune response that follows. Therefore, the difference between tumor growth pattern observed in heterozygous Ncr1\(^{gfp/+}\) and NCR1-deficient Ncr1\(^{gfp/gfp}\) mice could be due to differences in either T cell responses or to a direct NK cell response. To further establish the role played by NK cells in the PD1.6 killing, PD1.6 s.c. tumor growth was monitored in NK-depleted and nondepleted mice. Heterozygous Ncr1\(^{gfp/+}\) mice were i.v. injected with 50 \( \mu \)g of anti-NK1.1 Ab into the tail vein. Sterile PBS was injected as a negative control. We have chosen to

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**FIGURE 4.** NK and T cells are detected within tumors. A, Flow cytometry analysis of PD1.6 cells using PE-conjugated anti-CD3 mAb. Gray shading, background staining with secondary Ab only; dark line, specific staining. Data from one of three independent experiments are shown. B and C, Detection of immune cells within tumors. NK and T cells were detected within tumors derived from either three Ncr1\(^{gfp/+}\) (B) or three Ncr1\(^{gfp/gfp}\) (C) mice at day 11 after injection of PD1.6 tumor cells. Data from one of two independent experiments are shown.
use heterozygous mice for this experiment because all of their NK cells specifically express a GFP reporter (37) that can easily be visualized by flow cytometry and, importantly, they have been shown to be normal and to behave as wild-type mice (37). To verify NK cell depletion, mice were bled 2 days after injection and cells were analyzed by flow cytometry to detect GFP-expressing NK cells. Blood from a wild-type Ncr1<sup>+/−</sup> mouse that does not carry a GFP reporter was used as a reference. As can be seen in Fig. 3A, ~1% of GFP-positive cells were detected in blood derived from untreated heterozygous mice, accounting for peripheral blood NK cells, whereas no GFP-positive cells were detected in blood derived from NK-depleted mice.

Next, 1 × 10<sup>6</sup> PD1.6 tumor cells were injected s.c. into NK-depleted and nondepleted mice, and tumor growth was monitored every 2 days for a period of 12 days. In agreement with the above results, in the NK-depleted mouse group, tumors were visible beneath the skin by day 3 and were measurable by day 4, whereas tumors were visible only by day 7 in the nondepleted mouse group. In both mouse groups, tumors were gradually growing until day 10 when they started to decrease and the experiment was terminated due to loss of NK depletion (depletion was verified every 2 days during the experiment and on day 10 NK cells emerged in the depleted mice). Over the entire period of the experiment, tumors were larger in the NK-depleted mouse group (Fig. 3B). Thus, NK cells in general and NCR1 in particular play an important role in the in vivo killing of PD1.6.

Detection of NK cells within tumors

The above experiments show that the function of NK cells in general and Nkp46/NCR1 in particular is critical between days 5 and 11, the time window in which tumors start growing. To test whether NK cells and T cells are present within tumors at these time points and to determine whether the “tumor-enhancing” effect observed in the Nkp46/NCR1 knockout mice was associated with a decreased migration of NK cells or T cells, we used the Ncr1<sup>1gfp/1gfp</sup> and the heterozygous Ncr1<sup>1gfp/+</sup> mice. Mice were s.c. injected with 1 × 10<sup>6</sup> PD1.6 tumor cells and sacrificed at day 11 after injection (the same day in which tumor growth reached its maximum in the heterozygous Ncr1<sup>1gfp/+</sup> mice (Fig. 2)). Tumors were removed and lymphocytes in the tumors were quantified by flow cytometry. NK cells were visualized using the GFP they specifically express and T cells were identified by staining with anti-CD3 mAb. Importantly, PD1.6 cells are a thymoma lines that lacks expression of CD3 on their cell surface in vitro (Fig. 4A). To verify that PD1.6 cells do not express CD3 even after in vivo growth, that would complicate the detection of migrating T cells, heterozygous Ncr1<sup>1gfp/+</sup> and Ncr1<sup>1gfp/1gfp</sup> mice were s.c. injected with 1 × 10<sup>6</sup> PD1.6 tumor cells. Mice were sacrificed at day 9 after injection (when tumors were large enough to allow their removal), tumors were removed, and tumor content was analyzed by flow cytometry for the binding of NCR1-Ig (to detect the PD1.6 cells and to determine whether the in vivo tumor growth of other lymphoma cell lines such as B10 and EL4 that were recognized by NCR1 and NKP46-Ig (Fig. 1, A and B) but were not able to efficiently activate the BW reporter system (Fig. 1E). Wild-type Ncr1<sup>1gfp/+</sup> or Ncr1-deficient Ncr1<sup>1gfp/1gfp</sup> mice were injected s.c. with 1 × 10<sup>6</sup> B10 or EL4 tumor cells and tumor growth was monitored every 1–2 days. Similar to PD1.6, both in B10 (Fig. 5A) and in EL4 (Fig. 5B) tumor growth was observable beneath the skin by day 6 and measurable by day 8 after injection in both Ncr1<sup>1gfp/+</sup> mice and Ncr1<sup>1gfp/1gfp</sup> mice. However, in marked contrast to PD1.6 tumor growth, no significant difference in the in vivo growth of either B10 or EL4 tumors was observed in the absence or presence of NCR1. In the case of B10 tumor growth (Fig. 5A), tumor size peaked at day 8 after injection, diminished thereafter, and was not visible by day 11 after injection in both groups of mice. EL4 tumors (Fig. 5B); on the other hand, were detectable until day 14 after injection, peaking at day 12, and reached bigger sizes than B10 tumors did.

FIGURE 5. NCR1 does not play a role in the in vivo B10 and EL4 tumor growth. Tumor size was monitored every 2 days after s.c. injection of 1 × 10<sup>6</sup> B10 tumor cells (A) or EL4 tumor cells (B) in 6 wild type Ncr1<sup>1gfp/+</sup> mice and 6 NCR1-deficient Ncr1<sup>1gfp/1gfp</sup> mice. Data from one of three independent experiments are shown.

B10 and EL4 in vivo tumor growth is not altered in the absence of NCR1

To test whether the BW screening assay could indeed select for tumors expressing functional ligands for NCR1, we also examined the in vivo tumor growth of other lymphoma cell lines such as B10 and EL4 that were recognized by NCR1 and NKP46-Ig (Fig. 1, A and B) and were not able to efficiently activate the BW reporter system (Fig. 1E). Wild-type Ncr1<sup>1gfp/+</sup> or Ncr1-deficient Ncr1<sup>1gfp/1gfp</sup> mice were injected s.c. with 1 × 10<sup>6</sup> B10 or EL4 tumor cells and tumor growth was measured every 1–2 days. Similar to PD1.6, both in B10 (Fig. 5A) and in EL4 (Fig. 5B) tumor growth was visible beneath the skin by day 6 and measurable by day 8 after injection in both Ncr1<sup>1gfp/+</sup> mice and Ncr1<sup>1gfp/1gfp</sup> mice. However, in marked contrast to PD1.6 tumor growth, no significant difference in the in vivo growth of either B10 or EL4 tumors was observed in the absence or presence of NCR1.

These results indicate that NK cells and T cells enter tumors during growth and are both present by day 11 after injection of cells in equal numbers in both mouse strains. Moreover, the fact that no difference in NK cell percentage between NCR1-deficient Ncr1<sup>1gfp/1gfp</sup> and Ncr1<sup>1gfp/+</sup> mice was observed indicates that NCR1 does not play a role in the trafficking of NK cells in tumors in vivo. Similar results were observed with influenza infection (37).
Direct killing of PD1.6 lymphoma cells is mediated by NKP46/NCR1 receptor

Activation of NK cells by target cells can result in direct cytotoxicity and/or in production of cytokines such as IFN-γ and TNF-α (18). Therefore, we next examined the functional involvement of NK cells in the immune-mediated control of tumor growth. To test whether NCR1 is directly involved in the direct killing of PD1.6 tumor cells, we used an in vitro cytotoxicity assay in which NK cells from heterozygous Ncr1+/−/gfp and NCR1-deficient Ncr1−/−/gfp mice were incubated with various tumor cell lines (indicated in the x-axis) and anti-CD107a Ab. CD107a levels on GFP-marked NK cells were quantified using flow cytometry analysis. Values are mean ± SD for duplicate samples. B and C, Reduced in vivo killing of PD1.6 cells in Ncr1−/−/gfp mice. Vybrant Dil-labeled PD1.6 cells and Vybrant DiD-labeled HeLa cells were injected into the tail vein of three wild-type Ncr1+/+ mice and three NCR1-deficient Ncr1−/−/gfp mice. Cells in the lungs (B) were quantified 3 h after injection using flow cytometric analysis. Data from one of three independent experiments are shown. C, PD1.6 cells were labeled as above and cells in various organs (indicated in the x-axis) were monitored either immediately after the injection or 3 h later. D, IFN-γ secretion of poly(I):poly(C)-activated NK cells from mice of different genotypes (key) in response to a 48-h incubation with different target cells. Values are mean ± SD for duplicate samples. Data from one of three independent experiments are shown.

**FIGURE 6.** Direct killing of PD1.6 lymphoma cells is mediated by the NKP46/NCR1 receptor. A, Poly(I):poly(C)-activated NK cells were isolated from heterozygous Ncr1+/−/gfp and NCR1-deficient Ncr1−/−/gfp using the AutoMACS instrument and incubated for 2 h with various tumor cell lines (indicated in the x-axis) and anti-CD107a Ab. CD107a levels on GFP-marked NK cells were quantified using flow cytometry analysis. Values are mean ± SD for duplicate samples.

**A** and **B**, NK cells from Ncr1+/+ mice were activated in vitro with Poly(I):poly(C) and were tested for cytotoxicity in a 4-h assay against PD1.6. IFN-γ production was also measured in vitro. **C**, NK cells from Ncr1+/+ or Ncr1−/−/gfp mice were activated in vitro with Poly(I):poly(C) and were tested for cytotoxicity in a 4-h assay against PD1.6. IFN-γ production was also measured in vitro. **D**, NK cells from Ncr1+/+ or Ncr1−/−/gfp mice were activated in vitro with Poly(I):poly(C) and were tested for cytotoxicity in a 4-h assay against PD1.6. IFN-γ production was also measured in vitro.

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The positive control YAC-1 lymphoma cells. A marked decrease in CD107a expression was observed when PD1.6 tumor cells were incubated with NCR1-deficient NK cells derived from Ncr1−/−/gfp mice, indicating that the interaction between PD1.6 cells and NK cells via NCR1 in vitro leads to direct cytotoxicity. In contrast, much less killing was obtained when NK cells were incubated with B10 and EL4 tumor cells, as percentages of CD107a-positive NK cells were almost as low as that of the negative control and no difference was seen between Ncr1−/−/gfp- and Ncr1+/+ derived NK cells. Thus, NCR1 is not critically involved in the killing of B10 and EL4 and the general ability of NK cells to kill these two targets is poor. The reduced killing of PD1.6 tumors was due to the absence of NCR1 and not due to a general reduction in NK cytotoxicity of the NCR1-deficient Ncr1−/−/gfp mice because similar levels of YAC-1 killing were observed when either Ncr1−/−/gfp- or Ncr1+/+ derived NK cells were used.

These combined in vivo and in vitro results indicate that the killing of B10 and EL4 tumor cells is not NCR1 dependant because these lymphoma cell lines were efficiently rejected by both wild-type and NCR1-deficient mice, were less killed in vitro by
NK cells, and were less recognized by the NCR1 using the BW reporter assay. Thus, immune cells other than NK cells probably play a more significant role in the rejection of these two cell lines. Indeed, several works have shown that both B10 and EL4 lymphomas are subject to T cell-mediated antitumor immune responses (48, 49).

To further reinforce the results obtained in vitro, we used an in vivo cytotoxicity assay, a lung tumor clearance assay with minor modifications owing to the presence of GFP in the mice (37, 45). In this assay, fluorescence-labeled tumor cells are injected i.v. into the tail vein of mice, and cells that reach the lungs get trapped in the lung’s capillary blood vessels where they are subject to immune cell-mediated attack. Taken into account the short-term duration of the experiment along with no previous exposure of the mice to the injected tumor cells, such an experiment probably says in vivo the involvement of innate immune cells such as NK cells in the killing of the injected cells. Because injection of the cells might vary between different mice and because we aimed to compare the ability between the Ncr1<sup>1gfp/gfp</sup> and wild-type Ncr1<sup>+/+</sup> mice to kill tumor cells in vivo, each injection was controlled using internal control HeLa cells, which are not killed by NK cells (Fig. 6A). HeLa cells were labeled using Vybrant DiD, PD1.6 target cells were labeled using Vybrant Dil, and 2 × 10<sup>6</sup> cells of each cell type were mixed and injected together into the tail veins of Ncr1-deficient Ncr1<sup>1gfp/gfp</sup> and wild-type Ncr1<sup>+/+</sup> mice. Lungs were explanted 3 h later and the amount of labeled cells was quantified by flow cytometry. We observed that PD1.6 cells were less cleared from the lungs in Ncr1<sup>1gfp/gfp</sup> mice compared with their lung clearance in Ncr1<sup>+/+</sup> mice: the ratio of PD1.6:HeLa was ∼0.5 in Ncr1<sup>+/+</sup> mice but was ∼0.95 in Ncr1<sup>1gfp/gfp</sup> mice, meaning that the in vivo killing of PD1.6 cells was impaired in Ncr1<sup>1gfp/gfp</sup> mice compared with their killing in Ncr1<sup>+/+</sup> mice (Fig. 6B).

To test whether tumor cells undergo clearance in other organs, 2 × 10<sup>6</sup> Vybrant Dil-labeled PD1.6 cells were i.v. injected into the tail vein of wild-type mice. Lungs, spleens, livers, inguinal lymph nodes, and kidneys were harvested either immediately after injection or 3 h later, and the amount of labeled cells in these organs was quantified by flow cytometry. The number of cells reaching the lymph nodes or kidney was very small (Fig. 6C). Tumor cell clearance was seen in the lungs and liver and was more pronounced in the lungs. Thus, the i.v. injected tumor cells rapidly appear in various organs and are subjected to killing by innate cells in these organs. Interestingly, in the spleen, there was an increase in tumor cell counts along the course of the experiment (Fig. 6C). One possible explanation for such an increase is that the spleen directly captures Ags from the blood and is highly populated with T cells (∼200 × 10<sup>6</sup>). The amount of NK cells in the spleen is only ∼0.5%. Thus, it might be possible that the PD1.6 tumor cells in the spleen are inaccessible for NK cells due to the huge excess of the T cells.

NK cells may also have an indirect effect on the control of tumor growth by cytokine secretion and/or regulation of the adaptive immune response. To test whether the interaction of NK cells with PD1.6 tumor cells can cause IFN-γ secretion, NK cells were extracted from poly(I):poly(C)-activated splenocytes of both wild-type and Nkp46/NCR1 knockout mice. NK cells were incubated with PD1.6 cells or control mouse tumor cell lines. Supernatants were collected 48 h later and examined for IFN-γ levels by ELISA. The RMAS tumor cell line, which has been previously shown to express a ligand for NCR1 (37), and the HeLa cell line, which has been shown not to express such a ligand (Fig. 6A), were used as positive and negative controls, respectively. As demonstrated in Fig. 6D, no significant IFN-γ secretion above basal levels (no targets) was observed in response to incubation with PD1.6 cells, as compared with the RMAS cell line. Taken together, these results demonstrate that the interaction between NK cells and PD1.6 lymphoma cells, via NCR1, does not lead to IFN-γ secretion rather than to direct cytotoxicity.

**Discussion**

In recent years, the immune surveillance hypothesis of tumor immunity, once dismissed, is on the rebound. Data from induced mutant mouse models lacking distinct immune cell populations or effector molecules clearly demonstrate an increased incidence of spontaneous tumors as well as a higher susceptibility to induced and transplanted tumors (2, 3, 23, 24, 26, 27).

NK cells represent a highly specialized lymphoid population characterized by a potent cytolytic activity against tumor and virally infected cells (1, 7, 8, 14). A role for NK cells in the rejection of tumors had been proposed shortly after their discovery as a unique lymphocyte subset (4, 50) and has been established in many in vitro and in vivo studies since then. In the work presented here, we have decided to focus on the role of the NKP46/NCR1 receptor, a main activating NK cell receptor, in tumor recognition in vivo.

To assay for the presence of potent unknown ligands for NCR1, we have devised a BW-NCR1/ζ reporter system that is based on the fact that a self unknown ligand for NCR1 exists on BW cells causing continuous secretion of IL-2 due to constant triggering of the NCR1/ζ fusion receptor. Our hypothesis was that tumor cells expressing a significant amount of ligand(s) for NCR1 will induce an IL-2 secretion that would overcome the self-secretion of BW-NCR1/ζ cells. The PD1.6 lymphoma cell line was the only cell line tested that showed such an effect in the BW-NCR1/ζ assay.

A discrepancy was observed between the BW reporter assay (Fig. 1E) and the Ig fusion-binding assay (Fig. 1, A–C). Although all tumors (B10, EL4, and PD1.6) bind both NCR1-Ig and Nkp46-Ig to the same extent, only PD1.6 was able to induce a strong IL-2 secretion from the BW-NCR1/ζ cells. Few explanations may account for this phenomenon:

1. Differences in the glycosylation of a bound (BW assay) and soluble receptor (Ig fusion protein) could account for the differential binding capacity of putative ligands. If that is the case, using Ig fusion proteins might be problematic in some situations since both in vitro and in vivo assays demonstrated that indeed the killing of PD1.6 is NCR1 dependent and the killing of EL4 and B10 is not. These results suggest that the BW assay is superior and more reliable than the Ig-fusion proteins.

2. It is also possible that PD1.6 stimulates more effectively the BW-NCR1/ζ than B10 or EL4 because of the expression of molecules other than NKP46 ligands, e.g., adhesion molecules or ligands for other receptors. However, since we show that PD1.6 tumor growth in vivo is accelerated in the absence of NCR1, whereas the in vivo growth of B10 and EL4 is similar in the knockout and wild-type mice, we concluded that the BW system is indeed a more reliable assay as compared with the Ig fusion proteins to predict the in vivo and the in vitro outcome of killing of cells by a particular NK receptor whose ligands are unknown.

The nature of the cellular ligands for NKP46/NCR1 remains elusive, despite extensive research. We speculate that one of the reasons for that might be the inability to select for tumors which express sufficient amounts of ligands, maybe due to the use of Ig fusion proteins, which, as we show here, might be inefficient in some cases. We hope that the system described here will allow the identification of the tumor ligand for NCR1 in the future.
The only ligands for NKp46/NCR1 identified to date are viral HA (44). We have previously demonstrated that the sialylation of NKp46/NCR1 is required for its interaction with HA (14, 44). Interestingly, the PD1.6 lymphoma cell line was generated by intrathymic inoculation of the radiation leukemia virus (51) However, our unpublished data showed that the sialylation of NKp46/NCR1 is not needed for the recognition of PD1.6 cells. These results are in agreement with our previous work which demonstrated that tumor recognition is sialic acid independent (47).

We demonstrate that NCR1 is directly involved in the in vivo killing of PD1.6. Although we cannot completely exclude the possibility that NKp46/NCR1 is also indirectly involved in the recognition of PD1.6 cells in vivo, we think that this option is unlikely. Our results show that PD1.6 tumors are less cleared, probably by NK cells, in vivo in the absence of NCR1. In addition, we show in vitro that the killing of PD1.6 by NK cells is dependent on NCR1. In contrast, no difference in the secretion of IFN-γ between wild-type and NCR1-deficient NK cells upon incubation with the tumor cells was observed. Thus, we concluded that NCR1 is directly involved in the killing of PD1.6.

We demonstrate a window of opportunities for NK cell activity. Between days 7 and 13, NK cells in general and NCR1 between wild-type and NCR1-deficient NK cells upon incubation with PD1.6. In contrast, no difference in the secretion of IFN-γ by NK cells is in the control of initial tumor growth; if the NCR1 knockout mice are healthy and all T cell parameters activity. Between days 7 and 13, NK cells in general and NCR1-deficient NK cells upon incubation with PD1.6 are less cleared, probably by NK cells, in vivo in the absence of NCR1. In addition, we show in vitro that the killing of PD1.6 by NK cells is dependent on NCR1. In contrast, no difference in the secretion of IFN-γ between wild-type and NCR1-deficient NK cells upon incubation with the tumor cells was observed. Thus, we concluded that NCR1 is directly involved in the killing of PD1.6.

References


